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Effect of *Kokuto,* a Non-Centrifugal Cane Sugar, on the Development of Experimental Atherosclerosis in Japanese Quail and Apolipoprotein E Deficient Mice

Masashi INAFUKU[1], Takayoshi TODA[2], Takafumi OKABE[3], Koji WADA[4], Kensaku TAKARA[4], Hironori IWASAKI[5] and Hirosuke OKU[5]*

[1] *United Graduate School of Agricultural Sciences, Kagoshima University, Korimoto 1–21–24, Kagoshima 890–0065, Japan*

[2] *Department of Clinical Laboratory Medicine, School of Medicine, University of the Ryukyus Hospital, Uehara 207, Nishihara, Okinawa 903–0215, Japan*

[3] *Department of Bioproduction, Faculty of Agriculture, University of the Ryukyus, Senbaru 1, Nishihara, Okinawa 903–0213, Japan*

[4] *Department of Bioscience and Biotechnology, Faculty of Agriculture, University of the Ryukyus, Senbaru 1, Nishihara, Okinawa 903–0213, Japan*

[5] *Division of Molecular Biotechnology, Center of Molecular Bioscience, University of the Ryukyus, Senbaru 1, Nishihara, Okinawa 903–0213, Japan*

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***Kokuto,* a traditional cane sugar of Okinawa, has been reported to have antioxidative and lipid-lowering properties. In this experiment, we investigated the effect of three different kinds of *Kokuto* (KA, KB, and KC) on atherosclerosis in two different animal models: Japanese quail and apolipoprotein E deficient (apoE-/-) mice. Ingestion of *Kokuto* had no significant effect on the serum and liver lipid levels of Japanese quail. Dietary intake of atherogenic diet (AD) with KA and KB decreased the liver triglyceride level and body weight in apoE-/- mice. Quail fed on AD with KA developed less extent of lipid-containing aortic intimal thickening lesions than those fed on AD with sucrose. Dietary intake of AD with *Kokuto* or sucrose induced aortic atheromatous lesions in mice, but the extent of atheromatous lesions was roughly comparable between these dietary groups of apoE-/- mice. The present study suggests that *Kokuto* prevents lipid-containing aortic intimal thickening lesions in Japanese quail.**

Keywords: *Kokuto,* atherosclerosis, Japanese quail, apolipoprotein E deficient mice

Introduction

Recently, atherosclerosis has been considered as the chief cause of morbidity and mortality in developed countries (Braunwald, 1997). More than 250 factors have been reported as risk factors associated with the development of coronary artery disease (Hopkins *et al.,* 1981; Hackam *et al.,* 2003). Austin *et al.* (1998) revealed the correlation between the serum triglyceride level as a risk factor and atherosclerosis by meta-analyses of epidemiological studies. An elevated serum cholesterol level, and particularly low-density lipoprotein cholesterol, has been also been reported to be one of the risk factors for atherosclerosis (Boullier *et al.,* 2001). A number of studies have showed that oxidized LDL plays a critical role in an early event of atherosclerosis (Choy *et al.,* 2004) and also oxidative stress is associated with several risk factors for atherosclerosis (Runge *et al.,* 1999). Therefore, the importance of lowered serum lipid levels and suppressed oxidative stress has been established to be beneficial in preventing atherosclerosis (Wierzbicki *et al.,* 2003; Kaliora *et al.,* 2005).

Kokuto, a non-centrifugal cane sugar of Okinawa, Japan, has been manufactured by a traditional process of boiling sugarcane juice. Sugarcane cultivated in tropical and subtropical environments grows under various kinds of oxidative stress, therefore it is expected that *Kokuto,* as well as sugarcane juice, contain many antioxidants such as phenolic compounds. Several investigators have reported that *Kokuto* contains a lot of new, in addition to previously known, antioxidants (Takara *et al.,* 2002). Non-sugar fraction derived from crude black sugar has been reported to inhibit the increase of serum TG level in rats (Kimura *et al.,* 1982). It has been already reported that Okinawan sugar cane rinds and wax decrease the serum cholesterol level in rats fed on the diets containing 1% cholesterol (Sho *et al.,* 1981 and 1984; Fukuda *et al.,* 1986). As mentioned above, *Kokuto* is expected to possess anti-atheorsclerotic functions by exerting lipid-lowering effects and antioxidative activities. However, the effect of *Kokuto* on the development of atherosclerotic lesions has never been studied. Thus, the objective of this study was to investigate the effects of *Kokuto* on lipid metabolisms, epididymal fat accumulation, and the development of atherosclerotic lesions in two different animal models: Japanese quail and apolipoprotein E deficient mice.

* To whom correspondence should be addressed.
E-mail: okuhiros@comb.u-ryukyu.ac.jp

Materials and Methods

Animal treatments All animals ethically approved under the rules and regulations of the Animal Welfare Center, University of the Ryukyus, Okinawa, Japan, were kept in specific pathogen-free conditions with laminal airflow and humidity. All animals were individually kept in cages at room temperature (25℃) and ambient lightening was automatically controlled to provide 12-h light and dark cycles.

Phenolic contents and antioxidative activities in Kokuto In this study, three different samples of *Kokuto* prepared in different factories (*Kokuto* A, B, and C) were used. The total phenolic content of *Kokuto* was measured by the Folin-Denis assay method according to the method described by Tateyama *et al.* (1997). Briefly, the reaction mixture, containing 200 μL of *Kokuto* solution (2 g/100 mL water) with 200 μL of Folin-Denis reagent, 400 μL of saturated sodium carbonate solution and 3.2 mL water, was allowed to stand for 30 min at room temperature, and then its absorbance was measured at 700 nm by a spectrophotometer (UV160, Shimadzu Corp., Kyoto, Japan). The unit of total phenolic content was expressed as mg of (+)-catechin/100 g of *Kokuto*. The DPPH scavenging activity of the *Kokuto* was examined according to the method described by Oki *et al.* (2001). Briefly, 300 μL of *Kokuto* solution (1 g/100 mL-4 g/100 mL), 300 μL of 20% EtOH, and 300 μL of 0.2 M-morpholinoethanesulfonic acid (MES) buffer at pH 6.0 were placed in a test tube. The reaction was initiated by adding 300 μL of 400 μM DPPH in EtOH. Following that, the reaction mixture was left to stand for 20 min at room temperature and its absorbance at 525 nm was measured by a spectrophotometer. The unit of DPPH scavenging activity was expressed as μmols Trolox equivalent/g of *Kokuto* using the standard Trolox curve.

Grouping and feeding of quailsquail A total of 40 Japanese male quail (*Coturnix japonica,* 2-month old) were purchased from Tokaiyuki Co., Ltd. (Aichi, Japan). The birds were randomly divided into a control (CO) diet group and three different *Kokuto* diet groups consisting of *Kokuto* A (KA), *Kokuto* B (KB), and *Kokuto* C (KC) diet group. The *Kokuto* diets consisted of 63% basal commercial diet (Kyoei Co., Ltd, Okinawa, Japan), 30% *Kokuto,* 5% corn oil, and 2% cholesterol. For the CO diet, 30% *Kokuto* was substituted with 30% sucrose. During the course of experimentation, all birds were strictly pair-fed on the same amount of the diets and given water ad libitum. At the 12th week of the experimental period, blood serum and liver specimens, and the entire aorta with its branches along with the heart, were collected from each bird for lipid analysis and to assess the degree of aortic lesions, respectively.

Grouping and feeding of mice To examine the effect of *Kokuto* on atherosclerosis in mice, apolipoprotein E deficient (apoE-/-) mice with BALB/c genetic background (BALB/c.KOR-ApoE^{shl}) were purchased from Japan SLC Inc. (Shizuoka, Japan). A total of 28 mice were randomly divided into CO, KA, KB and KC diet group that consisted of 6, 7, 7, and 7 mice, respectively. Each mouse diet was made on the basis of AIN-76 diet composition as shown in Table 1. Each experimental mouse was pair-fed on the diet and allowed access to water ad libitum through the entirety of the experimental period. All mice were euthanized at the 16th week of the feeding period. Blood serum, liver and the entire aorta with its branches along with the heart specimens, were collected from each mouse. The same procedure was carried out with respect to the quail also. Epididymal adipose tissues were excised carefully and then weighed.

Table 1. The composition of experimental mice diets.

Material	(g)
Casein*	20.00
β-Corn Starch*	15.00
Cellulose*	5.00
AIN76 Mineral mix*	3.50
AIN76 Vitamin mix*	1.00
DL-Methionine	0.30
Choline bitartrate	0.20
Corn oil	15.00
Cholesterol	0.15
Sucrose or *Kokuto*	39.85
Total	100.00

Lipid analysis Serum total cholesterol (TC) and triglyceride (TG) levels were measured using the commercially available enzymatic kits (Wako Pure Chemical, Osaka, Japan).

Total lipids were extracted from the liver tissues using the chloroform-methanol (2:1, v/v) method as reported previously (Folch *et al.,* 1957). The level of the liver TC and TG was determined by Shoenheimer-Sperry (Sperry *et al.,* 1950) and Fletcher's method (Fletcher *et al.,* 1968), respectively.

Histological and Immunohistochemical examinations The excised heart and 1 cm long proximal portion of ascending aorta and its large branches from quail and mice were fixed in 10% buffered formalin. The paraffin-embedded blocks were prepared from formalin-fixed tissues and underwent sectioning into 4-micrometer thickness for histological and immunohistochemical examinations. For histopathological study purposes, the tissue sections were stained by hematoxylin eosin (H.E.), Mallory azan (M.A.), and elastica van Gieson (E.V.). Immunohistochemical study was also carried out on the tissue sections using the Envision system (Dako, Kyoto, Japan), for which a panel of primary antibodies such as α-smooth muscle actin (αSMA) (Dako) and CD44 (Novocastra Lab., Newcastle, UK) were used in this study. To reduce the non-specific background staining, the blocking of endogenous peroxidase activity was performed with 3% hydrogen peroxidase. The tissue sections were incubated with the primary antibodies, and then allowed to react with labeled dextran polymer. The sections were stained with activated 3, 30-deaminobenzidine-tetrahydrochloride (DAB) solution, followed by counter-staining with Mayer-hematoxylin. Washing of the sections with Tris buffer saline three times was done after each step. The sections were then studied by light-

Table 2. Nutrient contents, the phenolic contents, and antioxidative activities of *Kokuto*.

	Kokuto		
Ingredient†	KA	KB	KC
Carbohydrates*	91.1	89.1	90.4
Protein*	1.1	2.5	1.8
Fat*	0.1	0.0	0.1
Ash*	2.9	3.7	3.0
Moisture*	4.8	4.7	4.7
Phenolic content (mg/100g)	451.7 [a]	309.6 [b]	385.8 [c]
Antioxidative activity (μmol Trolox equivalent/100g)	446.5 [a]	328.0 [b]	414.9 [a]

Table 3. Energy density and intake in each diet group.

Animal	Group†	Energy density of diet (kcal/g)	Food intake (g/day)	Energy intake (kcal/day)
Japanese quail	CO	3.921	10.3 ± 0.37	40.4 ± 1.5
	KA	3.828	10.7 ± 0.32	41.0 ± 1.2
	KB	3.819	10.5 ± 0.37	40.1 ± 1.4
	KC	3.813	10.8 ± 0.33	41.2 ± 1.3
	P value (ANOVA)		0.839	0.958
apoE-/- mice	CO	4.344	2.76 ± 0.08	12.0 ± 0.3
	KA	4.220	2.85 ± 0.05	12.0 ± 0.2
	KB	4.209	2.84 ± 0.07	12.0 ± 0.3
	KC	4.201	2.88 ± 0.07	12.1 ± 0.3
	P value (ANOVA)		0.962	0.879

†CO: Control diet, KA: *Kokuto* A diet, KB: *Kokuto* B diet, KC: *Kokuto* C diet.

microscopy. To evaluate the degree of atherosclerosis, the total intimal thickness (I)/medial thickness (M) ratio was measured with three aortic segments from each bird (Fig. 2 and 3).

Statistical analysis Statistical analysis was performed using one-way ANOVA, followed by inspection of the differences between pairs of mean values by Tukey-Kramer's test. All statistical analyses were performed using the SAS statistical software program (SAS Institute, Tokyo, Japan).

Results

The phenolic contents and antioxidative activities in Kokuto (Table 2) Three types of *Kokuto* used in this study showed high phenolic content and antioxidative activity. The phenolic content and antioxidative activity were the highest with KA, and the lowest with KB. Sucrose contained no phenolic substance and showed no antioxidative activity.

Body weight, and serum and liver lipid profiles in each experimental group In both the quail and mice experiments, no significant difference in energy intake was noted between each diet group (Table 3).

Table 4 lists the growth and lipid level parameters for the quail experiment. There was no significant difference in body weight between each diet group. Liver TG levels of all *Kokuto* diet groups tended to be lower in comparison to that of the CO diet group.

Table 5 summarizes the results of the mice experiment. The body weight of the KC diet group was significantly lower in comparison to that of CO diet group. The differ-

Table 4. Body and liver weights, and serum and liver lipid levels of Japanese quail.

Group†	Number	Body weight (g)	Serum lipid (mg/dL)		Liver weight (g/100g BW)	Liver (mg/g liver)	
			TC	TG		TC	TG
CO	n = 10	103.8 ± 2.2	1997 ± 361	246.0 ± 32.5	3.04 ± 0.38	30.5 ± 1.9	20.2 ± 2.2
KA	n = 10	103.1 ± 2.2	1633 ± 340	156.6 ± 28.6	2.42 ± 0.16	24.4 ± 3.1	14.4 ± 1.4
KB	n = 10	106.3 ± 2.4	1872 ± 254	199.6 ± 28.6	3.31 ± 0.27	28.3 ± 5.2	13.6 ± 2.0
KC	n = 10	105.9 ± 2.5	1673 ± 300	186.6 ± 22.8	2.35 ± 0.14	23.0 ± 2.7	16.2 ± 1.2
P value (ANOVA)		0.772	0.832	0.147	0.065	0.393	0.053

Table 5. Body, liver and epididymal adipose tissue weights, and serum and liver lipid levels of apoE-/- mice.

Group†	Number	Body weight (g)	Serum lipid (mg/dL)		Liver weight (g/100g BW)	Liver (mg/g liver)		Epididymal fat (mg/100g BW)
			TC	TG		TC	TG	
CO	n = 6	31.8 ± 0.6 [a]	723 ± 122	138.9 ± 8.2	3.90 ± 0.23	10.7 ± 0.9	30.9 ± 1.9 [a]	3.52 ± 0.40
KA	n = 7	27.6 ± 1.0 [ab]	745 ± 160	116.6 ± 12.0	3.93 ± 0.28	9.4 ± 1.8	21.6 ± 1.6 [b]	2.35 ± 0.36
KB	n = 7	29.3 ± 1.0 [ab]	816 ± 117	123.0 ± 14.1	4.21 ± 0.22	13.6 ± 0.5	24.9 ± 2.8 [ab]	2.17 ± 0.46
KC	n = 7	25.8 ± 1.2 [b]	751 ± 126	93.8 ± 2.7	4.02 ± 0.32	12.8 ± 1.7	23.6 ± 1.4 [ab]	1.96 ± 0.43
P value (ANOVA)		0.015	0.313	0.224	0.823	0.171	0.033	0.100

Data shown as mean±S.E.
†CO: Control diet, KA: *Kokuto* A diet, KB: *Kokuto* B diet, KC: *Kokuto* C diet.
Different letters shows significant difference ($P<0.05$).

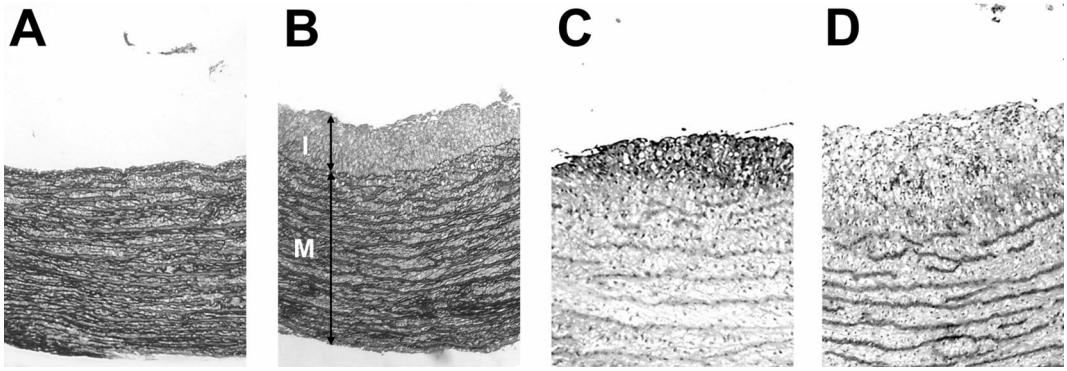

Fig. 1. Histological and immunohistochemical findings in the aorta of Japanese quail.
Elastica van Gieason stain of normal aorta (1A) and lipid-containing aortic intimal thickening lesion (LI) (1B). Lipid-containing cells in LI were positively stained with anti-CD44 antibodies (1C). Note positive reaction of anti-αSMA antibodies with smooth muscle cells in the aortic tunica media, but not with intimal cells in thickened intima in LI. (1D).
I: aortic tunica intima. M: aortic tunica media.

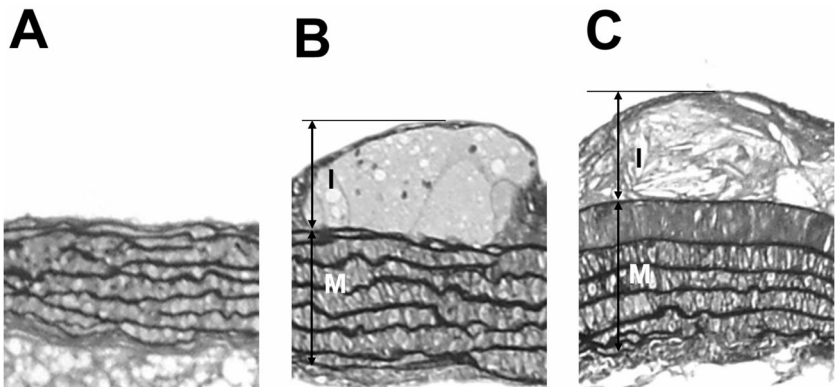

Fig. 2. Histological findings in the aorta of apoE-/- mice (elastica van Gieason stain).
Normal aorta (2A), lipid-containing intimal thickening lesion (2B), and developed atheromatous lesion (2C).
I: aortic tunica intima. M: aortic tunica media.

ence in liver TC levels was statistically insignificant between all experimental groups, whereas the liver TG levels were significantly lower in the KA diet group compared with that of CO diet group.

Histological and immunohistochemical findings Histological and immunohistochemical findings in Japanese quail are shown in figure 2. No significant intimal thickening lesions were observed in some birds (Fig. 1A), while significant atherosclerotic lesions were remarkable in most birds (Fig. 1B, C, and D). The most frequent type of atherosclerotic lesions were lipid-containing intimal thickening lesions (LI) which were consistent with the early stage of atherosclerosis (Fig. 1B). Lipid-containing cells in LI showed immuno-positive reactivity with anti-CD44 antibodies (Fig. 1C) but not with anti-SMA antibodies (Fig. 1D), suggesting the macrophages origin. The degree of aortic intimal thickening lesions (I/M ratio) of quail in the KA diet groups was significantly lower in comparison to the CO diet group (Fig. 3A).

Histological and immunohistochemical findings of apoE-/- mice are shown in Figure 2. A few mice had shown almost normal architecture of aortas (Fig. 2A), and lipid-containing intimal thickening lesions (Fig. 2B). Many mice developed atheromatous lesions with numerous cholesterin crystals that were compatible with the advanced stage of atherosclerosis (Fig. 2C). All dietary groups developed the atheromatous lesions to the same extent with an almost comparable degree of I/M ratio (Fig. 3B).

Discussion

Our present study reports the effect of dietary *Kokuto* intake on lipid metabolisms, epididymal fat accumulation, and the development of atherosclerotic lesions for the first time. Kimura *et al.* (1982) have previously reported that non-sugar fraction, derived from crude black sugar (*Kokuto*), inhibited the increase of serum TG levels in high sugar diet-fed rats. They further suggested that non-sugar fractions in black sugar inhibited the elevation of serum TG levels in rats fed on a high sugar diet by the reduction of both glucose and fructose absorption from the small intestine. Thus, the present result that the dietary intake of several types of Okinawan crude *Kokuto* decreased the liver TG levels in apoE-/- mice may support

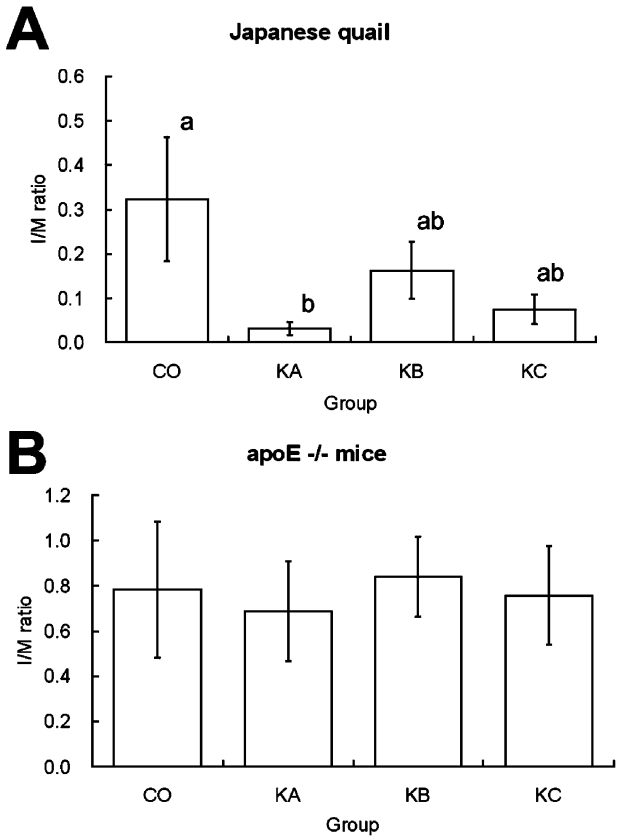

Fig. 3. The degree of atherosclerotic lesions in Japanese quail and apoE-/- mice.
CO: Control diet, KA: *Kokuto* A diet, KB: *Kokuto* B diet, KC: *Kokuto* C diet.
Different letters shows significant difference ($P<0.05$).

this previous observation. The accumulated reports of numerous researchers (Olefsky *et al.,* 1974; Parekh *et al.,* 1998; Leiber *et al.,* 2004) have suggested that the excess dietary intake of culinary fat and oil is associated with hypertriglyceridemia, obesity, and nonalcoholic steatosis. Serum and liver cholesterol levels have been reported to be decreased by dietary feeding of Okinawan sugar cane rinds and wax in rats (Sho *et al.,* 1981 and 1984). Fukuda *et al.* (1986) have examined the effects of wax, one component of sugar cane rinds, on the serum cholesterol level in lard fed rats and found insignificant effects of wax administration on the amount of fecal excretion of steroids in lard fed rats. They speculated that the cholesterol-lowering effect in sugar cane wax is due to the modification of cholesterol metabolisms instead of the fecal excretion of steroids. In the present experiment, *Kokuto* did not have any effects on the serum and liver cholesterol levels in both Japanese quail and in apoE-/- mice. Reliable evidence from animal model studies and the correlative data from human research have indicated that oxidative stress is the unifying mechanism for many atherosclerotic risk factors (Soccio *et al.,* 2005; Singh *et al.,* 2006). Steinberg *et al.* (1997) has suggested that the oxidation of lipoproteins, particularly low-density lipoprotein (LDL), is one of the important initial events in the pathogenesis of atherosclerosis. Therefore, antioxidants have been expected to be potentially useful therapeutic agents against atherosclerosis since they may inhibit lipoprotein oxidation involved in LDL, and reduce detrimental biological consequences caused by oxidative stress (Cynshi *et al.,* 2005). Moreover, many investigators have reported the beneficial effects of the anti-atherosclerotic functions of numerous types of antioxidants such as phenolic antioxidant AGI-1067, the mono-succinate ester of probucol, (Sundell *et al.,* 2003), BO-653, phenolic antioxidant (Cynshi *et al.,* 1998), and Vitamin E and/or C (Kaliora *et al.,* 2006). *Kokuto* has been recognized as a unique material in that it possesses various phenolic compounds and antioxidative activity (Takara *et al.,* 2003). Takara *et al.* (2003) illustrated that antioxidants isolated from *Kokuto* had phenolic hydroxyl groups in their structure, and suggested that radical-scavenging activity of these antioxidants is mostly related to the phenolic hydroxyl group. The different types of *Kokuto* used in this study contained various amount of phenolic compound. Japanese quail fed on a phenolic compound-rich type of *Kokuto* developed less severe atherosclerotic lesions than the control one, whereas apoE-/- mice fed on all types of *Kokuto* developed advanced atherosclerotic lesions. Recently, it has been reported by Kaliora *et al.* (2006) that the antioxidant therapy is supposed to be effective in the early stages of atherosclerosis by preventing LDL oxidation and oxidative lesion of endothelium. Most atherosclerotic lesions induced in our Japanese quail experiment were lipid-containing intimal aortic lesions consistent with the findings of the early stage of atherosclerotic lesions. On the other hand, apoE-/- mice have been known to be a very susceptible animal model to atherosclerosis and develop the atherosclerotic lesions at 9 weeks-old when they are fed on a low fat and sucrose diet without cholesterol (Jawien *et al.,* 2004). In our apoE-/- mice experiment, the most frequent type of atherosclerotic lesion was an atheromatous lesion that exhibits attributes similar to that of the advanced stage of atherosclerotic lesions. Furthermore, the species-dependant difference in susceptibility to atherosclerosis and the different predilection site of atherosclerotic lesions may exist in the anti-atherogenic effect of *Kokuto* as reported in probucol experiments (Daugherty *et al.,* 1991; Tardif *et al.,* 1993; Bird *et al.,* 1998). In conclusion, we demonstrate that *Kokuto* may be effective to prevent atherosclerosis in comparison with sucrose in Japanese quail. However, further study is required to clarify how *Kokuto* prevents the development of atherosclerotic lesions and improves the deteriorated lipid metabolism in different species.

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